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Relationship between the Physical Nature of Mitochondrial Membranes and Chilling Sensitivity in Plants [1, 2]

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Chilling injury in plants—the metabolic injury that occurs at low (0–10°) but not freezing temperatures—has been treated in descriptive studies (3, 9, 10, 11, 28), but little evidence has been developed on the basic nature of the injury. Lieberman et al. (14) examined the oxidative and phosphorylative activities (at 25°) of mitochondria derived from sweet potato roots which had been stored at 7.5 and 15°. Both oxidative and phosphorylative activities of the mitochondria from roots stored at the chilling temperature (7.5°) began to decline by the fifth week of storage, and the particles were completely inactive by the tenth week, but the activity of particles from nonchilled roots (15°) showed little change during this period. In contrast, Minamikawa et al. (19) reported that, while oxidative activity was less in mitochondria from chilled than from nonchilled sweet potato roots, there was no difference in the P/O ratios. It is difficult to reconcile these conflicting reports; however, in each case the highest P/O ratio of the control was only 1.0 to 1.5 (using α-ketoglutarate as substrate), indicating severe damage to the mitochondria during isolation procedures. Shichi and Uritani (25) worked with intact tissue and observed that sweet potato disks would not respond to dinitrophenol after the roots had been stored for 8 to 10 days at 0°. Furthermore, Lewis (11) found that chilling tomato fruit tissues for a short time at 0° decreased their subsequent incorporation of P^{32} at 20°, demonstrating an effect of chilling on phosphorylation.

Richardson and Tappel (23) recently demonstrated a difference in flexibility of mitochondrial membranes from warm-blooded animals as compared to those from cold-blooded animals. Using light-scattering technique to follow swelling, they showed that mitochondria of fish liver had the ability to swell at a rapid rate over a wide range of temperature down to 0°, but mitochondria from rat liver did not have this ability at the lower temperatures. They also showed that a correlation existed between membrane flexibility, as evidenced by mitochondrial swelling, and fatty acid composition of the mitochondrial membrane: the more flexible membranes from cold-blooded animals had a higher proportion of unsaturation in their fatty acids than did the less flexible membranes of the warm-blooded animals. The authors suggested that the increased flexibility made it possible for the mitochondria to carry on metabolic functions at lower temperatures.

Since metabolic studies with chilling-sensitive plant tissue have indicated that mitochondrial activity is affected by chilling temperatures, several plant species were examined to see whether a relationship between chilling sensitivity and membrane flexibility

[1] Revised manuscript received July 22, 1963.
[2] Based on a portion of a dissertation submitted by James M. Lyons to the Graduate Division, University of California, Davis, in partial satisfaction of the requirements for the Ph.D. degree, 1962. This investigation was supported in part by a research grant (EF-156) from the Division of Environmental Engineering and Food Protection, United States Public Health Service.
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similar to that observed in animals might exist in plants.

Materials and Methods

Plant Material. Mitochondrial preparations were made from the following chilling-resistant plant materials: cauliflower buds (*Brassica oleracea,* var. botrytis), turnip roots (*Brassica campestris,* var. rapa), and etiolated pea seedling epicotyls (*Pisum sativum,* cv. Morse's Progress No. 9). Chilling-sensitive materials studied were: tomato fruits (*Lycopersicon esculentum,* cv. UC 243–20), sweet potato roots (*Ipomoea batatas*), etiolated bean seedling hypocotyls (*Phaseolus vulgaris,* cv. Asgrow Kentucky 191), and etiolated corn seedling epicotyls (*Zea mays,* var. rugosa, cv. Asgrow Goldencross 51-T). Cauliflower, turnips, and sweet potatoes were purchased as needed from local markets; they were of high quality but of unknown cultivars. The sweet potato roots (yellow-fleshed Jersey type) were free from defects, but their previous storage history was unknown. Tomatoes for these studies were grown in a greenhouse. The tomato fruits were used when green and slightly immature or ripened on the vines until all green color had just disappeared. Bean, pea, and corn seedlings were grown in vermiculite, in a dark room, at 30° for 7 days.

Mitochondrial Preparations. Mitochondria were first prepared by standard methods (5, 13), using 1 low-speed centrifugation followed by 2 at high speed, but very little swelling of these particles could be detected, even when the suspensions were added to a hypotonic medium (distilled water). To seek an explanation for this inactivity, Janus Green B stain was employed as described by Potter et al. (21). In the presence of excess dye (1 ml of 0.1% dye in 40 ml of medium) all of the pellet material stains blue; but after incubation (35–40° for 5 minutes), only the mitochondria turn red. When cauliflower bud particles were prepared by the above centrifugation sequence, the resulting pellet contained 4 distinct layers, only one of which turned red during incubation. While this staining has been considered specific for mitochondria (18), Davies (2) reports some variability in the staining reaction. However, even if the technique does not rigorously establish the presence of mitochondria, it is still useful as a guide.

With use of the dye as a marker, the following improved centrifugation sequence was developed and gave a predominately red-stained final pellet: low-speed centrifugation (2000 × *g*) for 5 minutes, pellet discarded; high-speed (10,000 × *g*) centrifugation of supernatant material for 10 minutes, supernatant fraction discarded and pellet suspended in washing medium; low-speed (750 × *g*) centrifugation for 10 minutes, pellet discarded. This second low-speed centrifugation was critical, as it gave a pellet with a large amount of inert, nonstaining cellular debris. The new supernatant fluid was centrifuged at 10,000 × *g* for 10 minutes, and the pellet was suspended and washed twice more, recovering the mitochondrial fraction each time by centrifugation at 10,000 × *g* for 10 minutes.

All tissues were homogenized in a Waring blendor in the following medium: 0.25 M sucrose, 0.05 M Tris (pH 7.4), and 0.01 M EDTA. A ratio of 400 to 600 g of tissue to 1200 ml of medium was used. Pellets were washed in the same medium, lacking EDTA. The first washing (after the first high speed centrifugation) was done with about one third of the volume of medium used in the original homogenization, and in each successive washing the volume of medium was reduced by one half. The final pellet, consisting of 3 to 30 mg of protein nitrogen, was suspended in 1 to 2 ml of medium. Protein determinations were made colorimetrically with the Folin phenol reagent (15). To avoid injury by polyphenolic compounds during preparation of the sweet potato mitochondria (12), enough Carbowax 4000 (Union Carbide Chemical Corp.) was added to the initial homogenizing medium to give a content of 5%. This substance is effective in adsorbing and deactivating polyphenolic compounds during preparation of enzyme systems (Roy E. Young, private communication). To avoid injury from the highly acidic vacuolar contents during preparation of tomato fruit mitochondria, a pH electrode was inserted in the blendor cup during homogenization, and enough 1 N NaOH was added (1–2 ml) to keep the pH from falling below 6.5. The final brei was adjusted to pH 7.4.

Oxidative Activity. Oxidation of several acids of the tricarboxylic acid cycle by the mitochondrial preparations was observed with an O_2 electrode, constructed as described by Packer (20). The reaction mixture consisted of 0.25 M sucrose, 0.05 M Tris buffer (pH 7.4), 0.01 M substrate, 0.001 M $MgSO_4$, 0.01 M KH_2PO_4, 0.1 mg cytochrome c, 5×10^{-4} M CoA, and 0.2 ml mitochondrial suspension, in a total volume of 3.0 ml. O_2 uptake was followed for a period of 4 to 8 minutes at 20°.

Swelling Techniques. Mitochondrial swelling was measured at 25° by following light-scattering changes at 520 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer, as described previously for animal mitochondria (8, 23). The test solutions (each containing 0.05 M Tris buffer, pH 7.4) were: buffer only (hypotonic), 0.4 M sucrose (hypertonic), or 0.125 M KCl (approximately isotonic). To 3.0 ml of each of these solutions, 0.10 to 0.25 ml of mitochondrial suspension was added, giving an initial optical density between 0.40 and 0.52 in sucrose, and between 0.44 and 0.70 in KCl. These values correspond to 0.70 to 1.10 mg of protein nitrogen per tube.

Fatty Acid Analysis. Mitochondria were isolated as described above and stored frozen under nitrogen until methyl esters of their fatty acids were prepared. Saponification of the mitochondrial lipids and subsequent esterification were carried out by the methods of Marco et al. (17), using the modification they include for preparation on a microscale.

Table I

Oxidative Activity of Mitochondria at 20°.

These particles were samples of the same preparations used in the swelling studies of figures 1 and 2. Q_{O_2} (N) indicates the μl of O_2 consumed per mg protein nitrogen per hour. Values are reported for 2 tests of each kind of plant material, except as indicated. The reaction mixture consisted of 0.25 M sucrose, 0.05 M Tris buffer (pH 7.4), 0.01 M substrate, 0.001 M $MgSO_4$, 0.01 M KH_2PO_4, 0.1 mg cytochrome c, 5×10^{-4} M CoA, 0.2 ml mitochondrial suspension, final volume 3.0 ml.

Tissue	Q_{O_2} (N) for indicated substrate					
	Succinate		α-Ketoglutarate		β-Hydroxybutyrate	
Cauliflower bud	195	326	27	93	38	23
Pea epicotyl	530	286	278	38	45	19
Turnip root	234	306	23	36	29	32
Sweet potato root	364	192	124	46	32	21
Tomato fruit, dark pink	168	....	16	....	23	....
Tomato fruit, immature	198	....	45	....	16	....
Corn epicotyl	168	456	98	145	46	32
Bean hypocotyl	423	216	136	94	34	30

Gas chromatographic analyses of the methyl esters were made on an Aerograph Model A-90-C chromatograph with a 4-filament thermal conductivity detector connected to a Honeywell Electronik 1 mv strip-chart recorder. The analytical column was 8.5 feet of 0.25 inch O.D. copper tubing, packed with 20% diethylene glycol succinate on 60/80 mesh firebrick. The operating conditions were: temperature, 205°; helium flow rate, 80 ml/minute; and filament current, 235 ma. These conditions gave good separation of stearic, oleic, linoleic, and linolenic acids, which were of particular interest. Peaks were identified (i) by comparing their retention times with those of a known mixture of 7 esters of purified fatty acids, (ii) by comparing a semilogarithmic plot of relative retention time against chain length or degree of

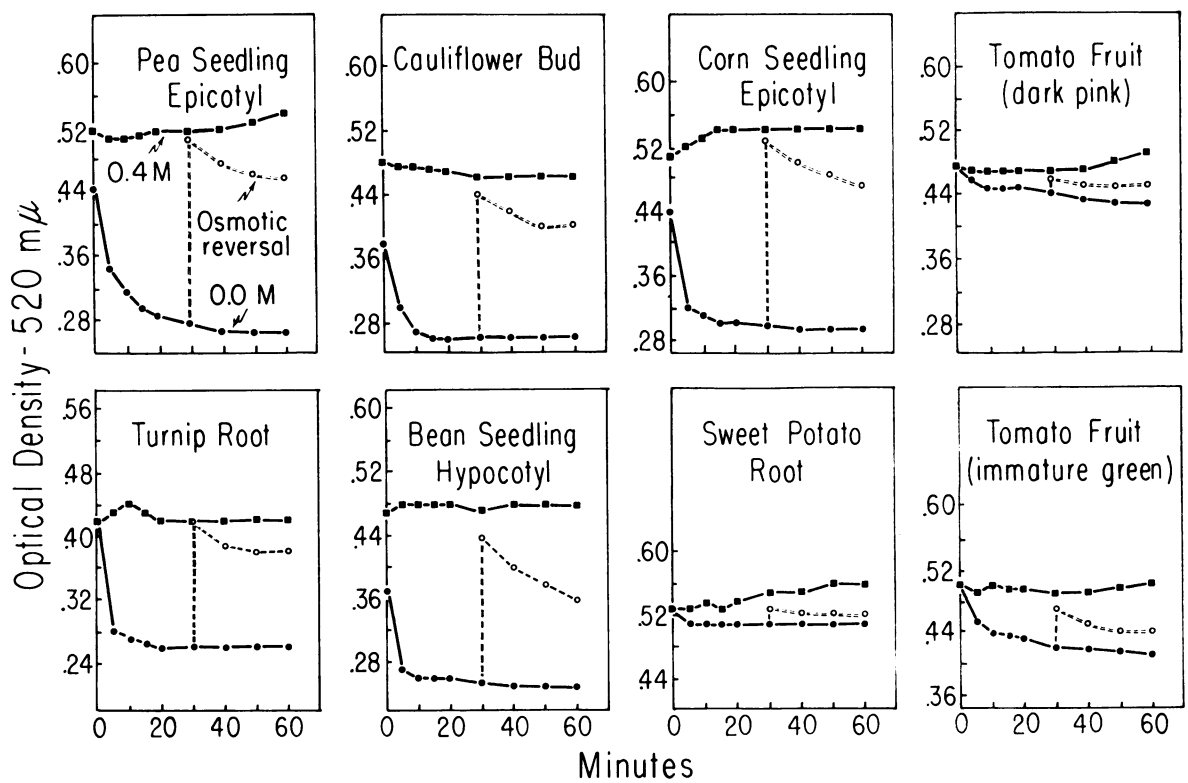

FIG. 1. Swelling of isolated plant mitochondria at 25° in hypotonic (no sucrose added) and hypertonic (0.4 M sucrose) buffer solutions as indicated by changes, at 520 mμ in the optical density of test solutions. Dotted line at 30 minutes indicates osmotic reversal of the hypotonic swelling, by addition of 1.0 M sucrose, to make the test solution 0.4 M with sucrose. Legends shown for pea seedling epicotyls apply to the same symbols in the other graphs.

unsaturation for the various components, and (iii) by comparing relative retention times with published data obtained under comparable conditions (6). Calculations were made in a manner similar to that of Smith (26).

Results

Mitochondrial Criteria. To establish the presence of functional mitochondria, the preparations were assayed for oxidative capacity and cytochrome content. Table I presents the rates of oxidation of succinate, α-ketoglutarate, and β-hydroxybutyrate. Although no attempt was made to study cofactor or other requirements for increased oxidative activity, the data show that the preparations had the capacity for these characteristic reactions of mitochondria. In addition, difference spectra of these preparations demonstrated the presence of cytochromes, in qualitative agreement with previous reports for plant mitochondria (1, 4).

Swelling Ability of Mitochondria. The ability of the mitochondria isolated from various plant tissues to swell in hypotonic media was demonstrated by a decrease in optical density of the test solutions. In figure 1, the changes in optical density are presented for both hypotonic and hypertonic media. The osmotic reversal shown when sucrose was added after 30 min in the hypotonic solutions provides evidence that the observed changes in optical density are in fact due to mitochondrial volume changes. The assumptions that this method does in fact measure swelling (27) are thus validated. The striking osmotic reversal observed in these studies contrasts with the results of Honda and Muenster (5), who indicated that mitochondria stressed by hypotonicity lost their ability to act as osmometers, as measured by light-scattering changes.

A substantial range in apparent membrane flexibility exists among the species tested (fig 1). The tissues whose mitochondria showed very little ability to swell were chilling-sensitive sweet potato root and tomato fruits. The chilling-resistant tissues (pea seedling, turnip root, and cauliflower bud) all had mitochondrial membranes with a striking ability to swell. In contrast, mitochondria from bean and corn seedlings could swell to this same extent, although these are chilling-sensitive species.

Figure 2 shows the results obtained with other portions of these same mitochondrial preparations when 0.125 M KCl, instead of sucrose, was used as the osmotic medium. The same range in response is apparent; again the nonswelling mitochondria were from chilling-sensitive tissues, while the chilling-resistant tissues clearly had more responsive mitochondrial membranes. The curves for bean and corn seedlings again show that these chilling-sensitive tissues also contain mitochondria capable of swelling. However, in this system, the shape of the swelling

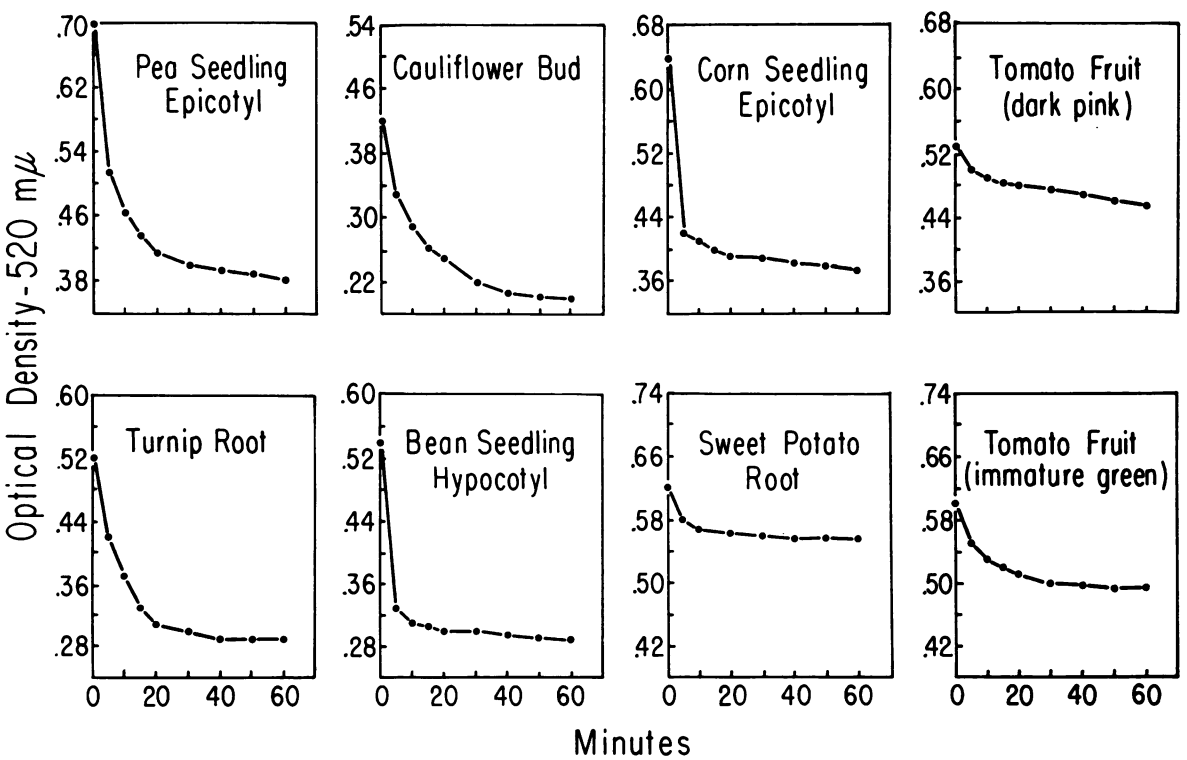

FIG. 2. Swelling of isolated plant mitochondria at 25° in 0.125 M KCl as indicated by changes, at 520 mμ, in the optical density of test solutions. Mitochondrial preparations are the same as those shown in figure 1.

curves for bean and corn seedling mitochondria clearly differs from the shape of the curves obtained with the chilling-resistant species, suggesting that the bean and corn mitochondrial membranes may have different properties which could be related to their chilling classification. The mitochondria from these 2 species show an exceptionally rapid osmotic adjustment within the first 5 minutes, but thereafter they show very little flexibility. In contrast, mitochondria from chilling-resistant tissues swell more gradually, requiring 20 to 30 minutes to reach the same state.

In table II, the data of figure 2 are further analyzed. The total difference in optical density between 5 and 60 min measures the extent to which mitochondrial swelling occurred during this period. This difference was 2 to 3 times greater in preparations derived from chilling-resistant tissues than in preparations from tissues sensitive to chilling. The rate constant (k), between 5 and 60 minutes, indicates a faster rate of swelling for mitochondria from chilling-resistant plants than for those from chilling-sensitive plants. The expression of initial optical density readings on a protein basis indicates that a reasonably uniform amount of mitochondria was present in each of the test solutions used for measurement of swelling.

The kinetics of mitochondrial swelling at different temperatures were tested with both chilling-sensitive and chilling-resistant species, as has been done with animal mitochondria (8, 20). There was little response over the range 0 to 40°, and an Arrhenius plot showed an essentially horizontal line, implying that swelling in these plant mitochondria is primarily a physical process.

Fatty Acid Analysis of Mitochondrial Membranes. Analyses of mitochondrial fatty acids from different plant tissues are presented in table III. The major acids found in the mitochondrial lipids were palmitic (16: 0), linoleic (18: 2), and linolenic (18: 3). Small amounts of oleic (18: 1), stearic (18: 0), palmitoleic (16: 1), and lauric (12: 0), and trace amounts of other unidentified fatty acids also were present. The double bond index shows a substantial range of values; as with mitochondrial swelling, the extreme values were shown by plants of markedly different resistance to chilling. The highest double bond indexes, indicating the greatest degree of unsaturation of fats, were shown by chilling-resistant cauliflower buds and turnip roots. The chilling-sensitive sweet potato roots and corn seedlings had the lowest indexes. Mitochondria from chilling-resistant pea seedlings and chilling-sensitive bean shoots and tomato fruits had indexes of intermediate values.

Discussion

The results presented in this paper show that the physical nature of the mitochondrial membranes of chilling-sensitive and chilling-resistant plants can differ. The greatest capacity for swelling, and the greatest degree of unsaturation of the membrane fatty acids, were shown by mitochondria from chilling-resistant species, and the lowest values for each of these criteria were shown by chilling-sensitive species.

Although complicated by intermediate values of these criteria, observed in some tissues from each category, the results suggest a parallel to the observations on mitochondria from cold-blooded and warm-blooded animals. Indeed, the range in percentage unsaturation of the mitochondrial fatty acids, as shown by the double bond indexes, between the extremes of chilling sensitivity of the plant tissues studied (table III) was greater than that shown by mitochondria from fish liver as compared to those from rat liver (23). However, the relative amount of fatty acids with any unsaturation at all may be as important a factor in membrane flexibility as the total number of double bonds. As shown in table III, not only is the degreee of unsaturation (DBI) generally

Table II

Swelling of Plant Mitochondria in KCl Solution

The test solution included 0.125 M KCl, 0.05 M Tris buffer, pH 7.4. Two tests were made with each tissue, except as indicated.

	% change in optical density 5–60 min		Rate constant (k)* 5–60 min		Initial optical density related to protein content**	
Chilling-resistant tissues:						
Cauliflower bud	0.14	0.13	0.05	0.06	0.620	0.640
Pea epicotyl	0.14	0.12	0.08	0.08	0.636	0.595
Turnip root	0.13	0.14	0.08	0.07	0.535	0.615
Chilling-sensitive tissues:						
Sweet potato root	0.03	0.04	0.04	0.03	0.615	0.565
Tomato fruit, dark pink	0.04	....	0.03	....	0.680	....
Tomato fruit, immature	0.05	....	0.05	....	0.615	....
Corn epicotyl	0.05	0.05	0.02	0.03	0.605	0.620
Bean hypocotyl	0.04	0.05	0.03	0.03	0.530	0.580

* k = reciprocal of the time to ¾ maximal swelling, which was taken as the optical density at end of 60 minutes.
** Optical density reading at 15 seconds divided by milligrams of protein nitrogen in the sample.

Table III

Fatty Acid Composition of Plant Mitochondria

Fatty acid**	Relative retention***	Per cent by weight of total fatty acid content*: Cauliflower bud	Turnip root	Pea shoot	Bean shoot	Sweet potato root	Corn shoot	Green tomato fruit
12:0	0.33	....	....	....	....	8.4	....	....
16:0	1.00	21.3	19.0	17.8	24.0	24.9	28.3	22.5
16:1	1.13	0.8	1.3	0.4	0.4	0.3	0.8	0.6
17:0	1.28	....	....	....	0.4	....	....	....
	1.48	....	0.4	....	0.2	....	0.4	0.6
18:0	1.68	1.9	1.1	2.9	2.2	2.6	1.6	2.5
	1.75	0.8	....	....	....	....	....	....
18:1	1.95	7.0	12.2	3.1	3.8	0.6	4.6	2.2
18:2	2.42	16.1	20.6	61.9	43.6	50.8	54.6	44.9
18:3	3.07	49.4	44.9	13.2	24.3	10.6	6.8	21.5
	3.20	....	....	....	....	....	0.7	....
22:0	4.98	....	....	....	....	....	1.5	....
Total weight per cent of C_{16} and C_{18} unsaturated acids		73.3	79.0	78.6	72.1	62.3	66.8	69.2
Total weight per cent of C_{12}, C_{16}, and C_{18} saturated acids		23.2	20.1	20.7	26.2	35.9	31.4	25.0
Ratio (unsaturated/ saturated)		3.2	3.9	3.8	2.8	1.7	2.1	2.8
Double bond index†		1.88±.21	1.89±.00	1.66±.11	1.64±.06	1.34±.09	1.34±.15	1.56±.12

* Averages of 3 preparations of mitochondria from each tissue.
** The ratio shown is the number of carbon atoms to the number of double bonds in the molecule.
*** Retention time relative to palmitic (16: 0) as 1.00.
† Double bond index (DBI): the summation of weight per cent of each acid multiplied by the number of double bonds it contains per molecule and divided by 100.

higher in chilling-resistant plants, but a higher percentage of the mitochondrial fatty acid content shows some unsaturation than is the case in the chilling-sensitive species, again suggesting a correlation between unsaturation and membrane flexibility. When Wheaton (28) determined the fatty acid content of whole root tissues of several chilling-sensitive and chilling-resistant species, his results in terms of the double bond index did not agree with these analyses of mitochondrial membranes, but the same trend in the ratios of unsaturated to saturated fatty acid contents could be discerned in his data. The significance of the relationship between fatty acid composition and relative flexibility of the mitochondrial membrane, as indicated by swelling studies, has been discussed in detail by Richardson et al. (23, 24).

Swelling phenomena of plant mitochondria differ from those observed in animal mitochondria (8, 16, 23). Plant mitochondria exhibit little spontaneous swelling in sucrose solution, as compared with animal mitochondria, but swell at a faster rate in KCl than do animal mitochondria. In addition, the rate of swelling of plant mitochondria is affected only silghtly by temperature, indicating that swelling in these studies was primarily a physical phenomenon, resulting from osmotic changes and diffusion of solutes. In contrast, the rate of swelling of isolated animal mitochondria is more responsive to temperature, implying enzymic or metabolic control of the swelling process.

That membrane flexibility can be related to the control of metabolic processes in the plant cell is a concept which deserves further study in relation to the mechanism of chilling injury. It is probably significant that protoplasmic streaming in chilling-sensitive plant cells stops abruptly when the temperature of the tissue is dropped below 10°, while streaming continues in cells from chilling-resistant plants almost to 0° (10, 28). As the temperature is lowered, the membrane system (plasma membranes, endoplasmic reticulum, mitochondrial membranes) of cells with a higher content of saturated fatty acids would become increasingly rigid in the chilling range (0–10°), whereas membranes with more unsaturation in their fatty acids could remain flexible down to 0°. A close correlation between oxidative phosphorylation and the swelling and contraction of mitochondria has been described by many workers (7, 22). If cellular membranes are unable to maintain a dynamic condition at low temperatures, the phosphorylative system could be disrupted and the supply of available ATP would be reduced. The effect of chilling would not necessarily be directly on the membranes or mitochondria themselves, but might depend on the length of time and the degree to which cells were without an energy supply adequate to maintain their normal functions. In contrast, plant tissues which are resistant to chilling temperatures possess membranes which apparently have a more flexible nature; such tissues should be able to carry out oxidative

phosphorylation and other normal cellular functions at the lower temperature.

Summary

Functional mitochondria were obtained from several plant species of differing sensitivity to injury at chilling temperatures (0–10°). The nature of the mitochondrial membranes was studied with light-scattering techniques and by fatty acid analysis. The following plants were studied: cauliflower buds, *Brassica oleracea,* var. botrytis; turnip roots, *B. campestris,* var. rapa; pea seedlings, *Pisum sativum;* tomato fruits, *Lycopersicon esculentum;* sweet potato roots, *Ipomoea batatas;* bean seedlings; *Phaseolus vulgaris;* and corn seedlings, *Zea mays,* var. rugosa.

A difference among plant species in properties of their mitochondrial membranes appears to be correlated with susceptibility of their tissues to chilling injury. Mitochondria from chilling-resistant tissue were more flexible, as measured by their greater ability to swell, than were mitochondria from chilling-sensitive tissue. Mitochondria from chilling-resistant species showed a higher content of unsaturated fatty acids than did mitochondria from sensitive species, and these observations may explain the differences in membrane flexibility. It is suggested that the metabolic injury caused in chilling-sensitive tissues may be due to inability of the relatively inflexible mitochondria to function at low temperatures.

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